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INTRODUCTION

Breast cancer is the most common malignancy in women in North America and is usually a disease of post-menopausal women (1). In the clinic, endocrine therapy is an important intervention in women with breast cancers that express estrogen receptor (ER). Treatment with tamoxifen and other antiestrogens has enhanced the survival of breast cancer patients, and these agents are now used in breast cancer prevention. The success of endocrine therapy in breast cancer is dependent on tight regulation of breast cell growth by steroid receptors (1, 2). However, as cancer progresses, it usually becomes resistant to estrogens, and most patients stop responding to tamoxifen or other antiestrogens. New findings on the role of an alternate estrogen signaling pathway in breast tumors may promote design of novel and more effective antihormone treatments for breast cancers (3).

Growth factor receptor malfunction also occurs in malignant progression, with members of the HER-1 (EGF) family frequently implicated in human cancer (1-3, 4-8). The HER (erb B) receptor family includes the HER-2 (erb B2) protein, a 185-kD transmembrane tyrosine kinase encoded by HER-2 oncogene (9-11), the HER-3 protein (12) and HER-4 receptor (13,14). Overexpression of HER-2 or related growth factor receptors is estimated to occur in two-thirds of sporadic breast cancers (1), while HER-2 amplification or overexpression is found in 25-30% of breast cancers in women and 41% of breast cancers in men (15-18). Overexpression of HER-2 is a marker of poor prognosis (15-19) and is associated with failure of antiestrogen therapy (3,20-31).

Receptors for estrogen occur in a family of potentially oncogenic receptors. Sequence similarities between the erb A gene product of avian erythroblastosis virus and ER suggest that these two proteins likely evolved from a common gene (32). Erb A genes cannot induce cell transformation alone, but cooperate with viral erb B oncogenes in cell transformation (33). With this lineage of cooperativity between erb A and erb B genes, it is not surprising to find reports of significant cross-talk and interaction between erb B (HER) pathways and ER signaling (3,24,27,34-36).

It is generally held that the biologic activity of estrogen in the breast is mediated through the specific high-affinity ER located in breast cell nuclei (1,37) [see FIG.1].

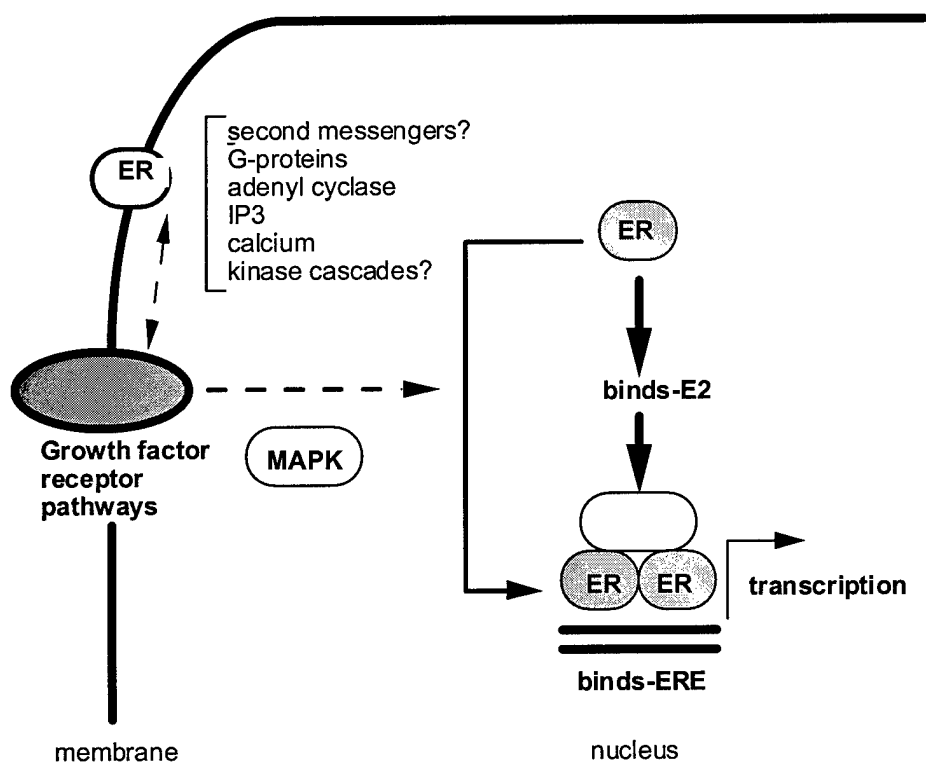


FIG. 1. Postulated cellular mechanism of action of estrogen (E2) and growth factors in breast cancers with estrogen receptor (ER). In most models of estrogen action, estrogen binding to ER in the nucleus promotes receptor dimer formation and receptor phosphorylation that enhances binding to nuclear estrogen-responsive elements (ERE) and coactivator proteins, leading, in turn, to initiation of gene transcription. However, this

model fails to account for numerous, rapid cell responses to estrogen (41-69). In the hypothesis to be tested here, estrogen may also bind to a membrane ER, with potential for stimulation of estrogenic responses via an alternate pathway. Current reports suggest that membrane-associated ER may activate one or more pathways, including interaction with growth factor membrane receptors such as HER-2 or activation of G-proteins, adenylate cyclase, inositol phosphate, calcium homeostasis and/or MAP kinase. These interactions may promote phosphorylation of ER via estrogen-induced activation of second-messengers and protein kinases or, alternatively, via ligand-independent pathways involving growth factor receptors. Growth of cells treated with estrogen may occur as a consequence of a synergistic feed-forward circuit where estrogen activates membrane signaling pathways that act, in turn, to enhance transcriptional activity of ER in the nucleus.

In the absence of estrogen, ER is considered to associate with proteins that prevent its interaction with the cell transcription apparatus. Upon estrogen binding, the receptor undergoes an activating conformational change that promotes association with target genes, thus permitting regulation of gene transcription [see FIG. 1]. In addition to the latter pathway, however, estrogen also induces rapid increases in levels of intracellular second messengers, including calcium (39,40) and cAMP (41,42), as well as activation of MAP kinase (43,44) and phospholipase (45). The timecourse of these events is similar to those elicited by peptides, lending support to the hypothesis that they do not involve genomic actions of estrogen. Both estrogens and growth factor ligands act as mitogens to promote cell growth in the breast, and the cellular effects of these agents sometimes overlap. The molecular details of this cross-talk between ER and erb B receptors are now beginning to emerge, and ER itself may be an important point of convergence (3,24,34-36).

Many of the rapid effects of estrogen are now attributed to the action of the hormone at the membrane, and these biologic actions appear to be mediated by membrane receptors that bind estrogen. The isolation and structural characterization of these native macromolecules have not yet been accomplished, and the derivation and functions of this receptor (or receptors) are largely unknown. Since activation of this alternate signaling pathway by estrogens may represent a mechanism by which estrogens regulate proliferation, we have investigated the nature and activity of this membrane response pathway in human breast cancer cells. Classical models of estrogen action that characterize this signaling pathway as solely due to the activity of an intracellular ligand-dependent transcription factor are clearly incomplete and must be modified to include estrogen receptors as significant components of other signaling pathways. As urged by others (40), "these data beg a reevaluation of the relative contributions of genomic and nongenomic activities in ER biology, an activity that is likely to support the development of pharmaceutical agents that exert differential activities in the two pathways".

RESEARCH PROGRESS

Aim 1) To assess the existence and identity of receptors for estrogen in plasma membranes of human breast cancer cells.

1.a. Enrichment of high-affinity binding-sites with specificity for E₂β in breast cancer cell plasma membranes

To confirm earlier reports of membrane binding-sites for E₂β (52,55,61,63), we measured specific [³H]E₂β binding in subcellular fractions of MCF-7 cells after controlled cell homogenization and fractionation (47,48). With recovery of more than 97% of total E₂β binding found in homogenates of MCF-7 cells, specific [³H]E₂β binding was distributed among crude nuclear, microsomal, mitochondria-lysosome and cytosol fractions (see Fig. 1 in ref. 84). After purification of plasma membranes from the crude nuclear fraction by use of discontinuous-sucrose density gradient centrifugation, the PM fraction showed enhanced activity of 5'-nucleotidase, a plasma membrane marker enzyme, to about 23-times that of homogenate. Specific [³H]E₂β binding in plasma membranes was enriched to 28-times homogenate activity and represented 22% of homogenate binding. This data shows that specific E₂β binding co-purifies with a plasma membrane marker protein in membrane fractions from breast cancer cells. LDH activity, highly enriched in cytosol, is not significantly detected in PM (84). In addition, cell DNA recovery was 94 ± 3 % of homogenate levels in nuclear fractions, and no DNA was detected in PM fractions (84).

Binding of [^3H]E $_2\beta$ by PM fractions from MCF-7 cells was analyzed further in equilibrium binding studies (see Figs. 2,3 in ref. 84). Samples of PM were exposed to [^3H]E $_2\beta$ concentrations ranging from 1×10^{-10} M to 5×10^{-9} M. Binding of hormone by PM is saturable, and Scatchard analyses of specific [^3H]E $_2\beta$ binding (cf. 48) show that the dissociation constant for the binding process is 3.6×10^{-10} M. Total binding sites in PM at saturation correspond to approximately 6.7 pmol E $_2\beta$ per mg membrane protein (84). In comparison with the estradiol binding properties of intact MCF-7 cells, plasma membrane estrogen-binding sites retain high affinity for specific estradiol binding and exhibit significant enrichment of ligand-binding capacity (see ref. 3). Further, ligand specificity of [^3H]E $_2\beta$ binding to PM was established by effective suppression by a 100-fold molar excess of unlabeled E $_2\beta$ (84). In contrast, [^3H]E $_2\beta$ binding by PM was essentially uninfluenced by these levels of estradiol-17 α , progesterone or testosterone (84). This portion of Aim 1 is now completed.

1.b. Identification of estrogen receptor forms in subcellular fractions after gel electrophoresis

To characterize putative estrogen receptor forms associated with PM fractions, samples were subjected to Western blot analysis, and blots were probed either with anti-ER antibody Ab2 or with E $_2\beta$ -POD (84). PM purified from MCF-7 cells show significant enrichment of a primary 67-kDa protein that reacts strongly with antibody Ab2 to LBD of nuclear ER- α (see Fig. 4 in ref. 84). Similarly, breast cell nuclear fractions are enriched with this protein reactive with ER- α (84). The 67-kDa band also shows evidence of specific labeling with E $_2\beta$ -POD (84). A secondary band at 46-kDa and minor bands at 62-kDa and 97-kDa were detected in PM and other cell fractions by use of Western blot and ligand-blotting (84). Using an antibody directed to ER- β , no significant reactivity with proteins at the expected size of 58-62 kDa was found in homogenate, nuclear or plasma membrane fractions of the MCF-7 cells (84). This portion of Aim 1 is now completed.

1.c. Purification of candidate receptors

As indicated above, work aimed at purification of candidate receptors is underway. As outlined in the original proposal, our efforts have involved the use of affinity chromatography, with recovered receptor to be used for preparation of monoclonal antibodies and for further molecular characterization and functional studies using cDNA for membrane ER. However, the yield of estrogen-binding receptor protein from purified plasma membranes has been very limited, and this strategy may require modification (85). In contrast, we have made very good progress in the isolation of membrane-associated ER from caveolae-related lipid raft subfractions of breast cancer cell plasma membranes (86), and this method may now allow further purification of membrane-associated receptor forms (87-91).

Aim 2) To assess the role of membrane estrogen receptors in promoting growth of breast cancers.

2.a. Rapid effects of E $_2\beta$ and E $_2\beta$ -BSA on activation of MAPK and Akt kinase in breast cancer cells

Post-receptor signal transduction events, such as stimulation of MAPK, extracellular signal-regulated kinase ERK-1 (p44) and ERK-2 (p42) (43,61), may contribute to proliferative effects of E $_2\beta$ in breast cells. Thus, we assessed estrogen-induced phosphorylation of MAPK in MCF-7 cells *in vitro*. E $_2\beta$, but not 17 α -estradiol (E $_2\alpha$), promotes phosphorylation of MAPK isoforms, with effects evident within 2 min (see Fig. 5 in ref. 84). To test whether activation of MAPK by E $_2\beta$ may be mediated by binding of estrogen to membrane-associated receptors, MCF-7 cells were treated with E $_2\beta$ linked to BSA, a macromolecular complex considered to be membrane-impermeant (52,61). Using E $_2\beta$ -BSA, but not control E $_2\alpha$ -BSA, phosphorylation of MAPK isoforms is again evident within 2 min of steroid administration. Incubation of cells with antibody against LBD of ER (Ab2) inhibited MAP kinase phosphorylation induced by E $_2\beta$ or E $_2\beta$ -BSA. Similarly, we assessed signaling via the phosphatidylinositol-3 kinase (PI3K)/Akt pathway after treatment of MCF-7 cells with E $_2\beta$ or E $_2\beta$ -BSA. Both ligands induced significant activation of Akt kinase (84), and inhibition of estrogen-induced effects occurred when cells were preincubated with ER antibody (Ab2), pure antiestrogen (ICI 182,780) or the PI3K inhibitor, LY 294002.

To assess the potential for MCF-7 cell activation by free estradiol liberated from E₂β-BSA, we transfected MCF-7 cells with an ERE-CAT reporter gene as before (3). Cells were exposed *in vitro* to free estradiol-17β or to E₂β-BSA for only 10 minutes, then washed and incubated further. After 24 hrs, ERE-CAT reporter gene activity was measured. Short-term treatment with free estradiol-17β stimulated a marked increase in reporter gene activity ($P < 0.001$), but E₂β-BSA elicited no significant effect (see Fig. 6 in ref. 84).

Since interaction of E₂β-BSA with plasma membrane binding-sites may be required for intracellular signaling (52,61), we evaluated binding of fluorescein-labeled E₂β-BSA (E₂β-BSA-FITC) in MCF-7 cells. E₂β-BSA-FITC binds at the surface of 77% of MCF-7 cells (see Fig. 7 in ref. 84), while only minimal background fluorescence is found among cells incubated with control ligand, BSA-FITC (84). In additional control studies, ER-positive ZR-75 breast cancer cells, as MCF-7 cells, show retention of E₂β-BSA-FITC at the cell surface, but ER-negative MDA-MB-231 breast cancer cells or COS-7 cells do not show significant binding of E₂β-BSA-FITC at the external membrane (84). On flow cytometric analysis (84), the E₂β-BSA-FITC complex shows evidence of ligand specificity, with significant reduction ($P < 0.01$) of E₂β-BSA-FITC binding by competition with equi-molar amounts of free E₂β, E₂β-BSA, tamoxifen or ICI 182, 780, while the related steroid congener, progesterone, is not effective. Surface binding of E₂β-BSA-FITC is significantly diminished by competition with antibody to LBD of nuclear ER, suggesting some immunologic identity of the membrane site with nuclear ER (84). As expected, after permeabilization of cells by disruption of plasma membrane with detergent, intense labeling of ER in cell nuclei is found and occurs in 96% of breast cancer cells (84). In other control studies, MDA-MB-231 cells with no ER showed no binding or retention of E₂β-BSA-FITC label, while ZR-75 breast cancer cells with ER expression did show surface binding of the complex (84). These portions of Aim 2 are now completed.

2.b. Inhibition of cell growth in vitro by antibody to ligand-binding domain of ER-α

Since antibodies to cell surface growth factor receptors are sometimes effective in blocking tumor growth (3,77), antiproliferative activity of antibodies to ER-α was evaluated using MCF-7 cells *in vitro*. The estrogen-dependent MCF-7 cells show enhanced growth after treatment with E₂β, but not E₂α (see Fig. 8 in ref. 84). However, prior exposure to LBD Ab1 or LBD Ab2 elicits a significant reduction ($P < 0.05$) in the E₂β growth response (84). Since some recent studies suggest that the proliferative response to E₂β is committed within 1 min and is evoked by activation of only a small fraction ($\leq 5\%$) of ER (73), we assessed the growth of breast cells after brief treatment with E₂β-BSA. MCF-7 cells were treated with 0.5 μM E₂β-BSA for only 10 min. Then, cells were rinsed and cultivated in estrogen-free media for an additional 72 h. The results show that E₂β-BSA ($P < 0.001$), but not control E₂α-BSA, stimulates cell growth (84). Moreover, the proliferative effect of E₂β-BSA is blocked by treatment of cells with ICI 182,780, a pure antiestrogen ($P < 0.001$) (84), or by prior exposure to anti-ER Ab1 ($P < 0.05$) or Ab2 ($P < 0.001$) (84). This portion of Aim 2 is now completed.

Aim 3) To investigate new treatment options to prevent breast cancer progression in human breast cancer.

3.a. Inhibition of breast tumorigenesis in vivo by antibody to ligand-binding domain of ER-α

The antitumor activity of antibodies to ER-α was evaluated further using MCF-7 tumors *in vivo*. MCF-7 cells were grown as subcutaneous xenografts in female athymic mice primed with E₂β to promote growth of these estrogen-dependent cells (3). Antibody or control treatments were initiated when tumors grew to $> 30 \text{ mm}^3$. Anti-ER Ab2 was administered in 6 doses over a 26-day period. The results show that antibody to ER, but not control immunoglobulin, elicits a significant suppression of tumorigenesis of human MCF-7 breast cancer xenografts in female nude mice treated concomitantly with E₂β (see Fig. 8 in ref. 84). This portion of Aim 3 is now completed.

3.b. Estrogen receptor interactions with growth factor membrane receptors

As noted above, activation of estrogen receptor-α (ER) by growth factors in the absence of estrogen is a well-documented phenomenon. To further study this process of ligand-independent receptor activation, COS-7

cells without ER were transfected with both ER and epidermal growth factor (EGF) receptor. In the absence of estrogen, EGF stimulated rapid tyrosine phosphorylation of ER in transfected COS-7 cells (see ref. 87). Similarly, in MCF-7 breast cancer cells that have natural expression of ER and EGF receptors, EGF promoted acute phosphorylation of serine and tyrosine residues in ER, and a direct interaction between ER and EGFR after treatment with EGF was found (87). In confirmation of direct interactions between ER and EGF receptors, activation of affinity-purified EGF receptor tyrosine kinase *in vitro* stimulated phosphorylation of recombinant ER (87). The cross-communication between EGFR and ER appears to promote significant stimulation of cell proliferation and a reduction in the apoptotic loss of those cells that express both receptor signaling pathways (87). However, COS-7 cells transfected with both ER and EGF receptors show minimal stimulation of classical estrogen response element (ERE)-dependent transcriptional activity after stimulation by EGF ligand. This suggests that the proliferative and antiapoptotic activity of EGF-induced ER activation may be dissociated from ERE-dependent transcriptional activity of the ER. Further consideration of the cross-communication between membrane-associated ER and membrane growth factor receptors, such as EGF and HER-2 receptors, may provide new targets for intervention in the clinic (88). In addition, new findings from our laboratory suggest that proximate interactions between membrane-associated ER and growth factor receptors may occur in specialized domains of plasma membrane, the caveolae-related lipid rafts (87-91).

PERSONNEL

During the past grant period, the following personnel were engaged on this project:

Richard J. Pietras, PhD, MD (PI)
Diana Marquez, MD (Postdoctoral Fellow)
Hsiao-Wang Chen, MS (SRA)

KEY RESEARCH ACCOMPLISHMENTS

- Plasma membrane-associated binding sites with high affinity and specificity for estradiol-17 β occur in human breast cancer cells.
- Plasma membrane-associated binding sites for estradiol in human breast cancer cells may play a role in modulating cell growth and survival.
- Plasma membrane-associated estrogen receptors interact with signaling initiated by membrane growth factor receptors
- Proximate interactions between membrane-associated ER and growth factor receptors may occur in caveolae-related lipid rafts of breast cancer cells

REPORTABLE OUTCOMES

Presentations

1. "Interactions between Type I receptor tyrosine kinases and steroid hormone receptors : Therapeutic implications". Presented at *First International Symposium on Translational Research in Oncology*, Dublin, Ireland (2001).
2. "HER-2 receptor signaling modulates estrogen receptor in breast cancer". Presented at Medical Oncology Seminar Series, UCLA School of Medicine (2001).
3. "Steroid and growth factor receptors: Cross-talk and clinical implications". Presented at *Second International Symposium on Translational Research in Oncology*, Anaheim, California (2002).
4. "Interactions between estrogen and growth factor receptors in human breast cancers and the tumor-associated vasculature". Presented at 8th Annual Multidisciplinary Symposium on Breast Disease, Amelia Island, Florida (2003).
5. "Estrogen receptor and human breast cancer therapy". Presented at Department of Pathology Seminar Series, UCLA School of Medicine (2003).
6. "HER-2 and estrogen receptor interactions in human breast cancer". Presented at Genentech BioOncology Herceptin Advisory Board Meeting, San Francisco (2003).

Abstracts

1. Marquez, D.C., Chen, H.-W. and Pietras, R.J. (2002). Membrane-associated estrogen receptors localize to caveola-related domains and contribute to growth regulation of breast cancer cells. DOD Breast Cancer Research Program Era of Hope Meeting Proceedings.
2. Marquez, D.C., Chen, H.-W. and Pietras, R.J. (2003). Estrogen receptor forms and HER-2/neu growth factor receptors co-localize in caveolae-related lipid rafts in human breast cancer cells. *Proc. Am. Assoc. Cancer Res.* 44 : 384.

Publications

1. Pietras, R.J., Nemere, I. and Szego, C.M. (2001). Steroid hormone receptors in target cell membranes. Endocrine 14 : 417-427.

2. Marquez, D.C. and Pietras, R.J. (2001). Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells. *Oncogene* 20: 5420-5430.
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5. Szego, C.M., Pietras, R.J. and Nemere, I. (2003). Plasma membrane receptors for steroid hormones: Initiation site of the cellular response. *Encyclopedia of Hormones* (in press).
6. Pietras, R.J. (2003). Steroid and growth factor receptors: Cross-talk and clinical implications. *In: Breast Cancer Management, 2nd Edition* (Reese, D., Nabholz, J.-M., and Slamon, D.J., editors), Lippincott, Philadelphia (in press).
7. Pietras, R.J. (2003). Interactions between estrogen and growth factor receptors in human breast cancers and the tumor-associated vasculature. *Breast Journal* (in press).

No abstracts, patents, degrees, development of cell lines, informatics or additional funding or research opportunities to be reported at this time.

CONCLUSIONS

A new approach to cancer therapy involves efforts to cut the lines of communication between hormone receptors and the cell nucleus, thus slowing or blocking cell division. Antiestrogen therapy is one well-known example of this approach, and it is often used to treat breast cancer and to prevent recurrence. Unfortunately, many patients do not respond to current therapy, and almost all treated patients eventually become resistant to antiestrogens. In addition, antiestrogens that are now available can result in abnormal uterine growth and thromboembolic events. The failure of antihormone therapy in the clinic is due to many factors, including the emergence of estrogen-independent growth that is no longer responsive to treatment with antiestrogen agonists.

New options for antiestrogen treatment are clearly needed, and alternative therapies may now derive from the current findings showing that ER molecules occur not only in the nucleus of the cell, but also in association with the surface membranes of human breast cancer cells. Moreover, these ER may interact with membrane HER-2 growth factor receptors. It is known that expression of HER-2 receptors occurs in many human breast cancers, and the enzyme activity of HER-2 may play a role in ER activation even in the absence of estrogen. If proximate interactions between ER and the HER-2 growth factor receptor occur and lead to promotion of cancer growth, this signaling axis may offer a new target for therapeutic intervention. Since overexpression of HER-2 in human breast cancers is associated with the failure of antiestrogen therapy in the clinic, understanding the biologic basis of the association between membrane ER and HER-2 receptors may help to improve decisions on patient management and to increase patient survival.

In the present work, we have made good progress in ascertaining the existence and nature of receptors for estrogen in surface membranes of human breast cancer cells. We have assessed the role of membrane ER in promoting growth of breast cancers. In challenging the dogma of estrogen action exclusively via an intracellular receptor, this work may lead to the development of previously unsuspected, less toxic antitumor therapies targeted to human breast cancer cells. A limited no-cost time extension would help us to complete all the goals of this proposal and to submit our findings for publication in peer-reviewed journals.

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